Growth of Bacillus sp. and Flavobacterium sp. in culture media with the addition of liquid whey tofu waste

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Abstract. The purpose of this study was to determine the growth of *Bacillus* sp. and *Flavobacterium* sp. when cultured together on the culture medium of whey tofu waste. This study used a Completely Randomized Design (CRD) with four treatments and five repetitions. The treatment was given by adding liquid whey tofu waste to a culture media in different doses; 0% (control), 10%, 20%, and 30%. The parameters observed in this study were the growth of *Bacillus* sp. and *Flavobacterium* sp. The results showed that the highest growth of *Bacillus* sp. and *Flavobacterium* sp reached 48 hours in the treatment with the addition of 10% liquid whey tofu waste with an average amount of 12.08×10^9 CFU / ml and 18.25×10^8 CFU / ml respectively. These were significantly different to the control ($P < 0.05$). The specific growth rate of *Bacillus* sp. and *Flavobacterium* sp. in the media with the addition of 10% liquid whey tofu waste was 0.11/hour and 0.13/hour respectively, which is higher than the control. The conclusion of this study was that the addition of the liquid whey tofu can increase growth and that the addition of 10% provides the best growth for the bacteria.

1. Introduction

The main problem that must be faced by farmers is the failure of production; this is one of the factors causing losses in shrimp farming. The prevention of diseases continues to be carried out but the farmers must still pay attention to the balance of the aquatic ecosystem. The control of the spread of diseases in shrimp culture in an environmentally-friendly manner (biological control) can be done using competitors for pathogenic bacteria [1].

The use of biological control is one of the better disease control strategies that allow for sustainable aquaculture can be created [2]. Competitor bacteria used as a biological control can be isolated from the bacterial habitat so then the bacteria are more adaptable and develop to suppress the pathogenic bacteria [3].

Flavobacterium sp. produces antibacterial compounds that can suppress the growth of other bacteria [3,5]. Another bacterium that can be used in fishery biotechnology is *Bacillus* sp. This is because *Bacillus* sp. has the ability to not produce toxins against shrimp that are maintained, easily grown, do not require expensive substrates and that have the ability to survive at high temperatures [6], thus it is widely used as a biocontrol agent [7]. *Bacillus* sp. can also produce antibiotics that can inhibit *Vibrio parahaemolyticus* [8] and several types of enzymes that have antagonism properties such as the carein enzymes produced by *Bacillus cereus* [9], the thuricin enzyme produced by *Bacillus thuringiensis* [10] and the bacillocin enzyme produced by *Bacillus subtilis* [11]. Based on the ability of

Flavobacterium sp. and *Bacillus* sp. when it comes to inhibiting the growth of pathogenic bacteria, it is necessary to conduct a bacterial culture or propagation to increase the availability of stock inoculants and applications in the field.

A bacterial culture is a way to multiply bacterial cells using a culture medium consisting of a mixture of nutrients [8,7]. The growth media must meet the nutritional requirements needed by a the microorganism, including carbon, nitrogen, vitamins, water and non-metallic elements such as sulfur, phosphorus and metal elements such as Zn, K, Cu, Mn, Mg, and Fe [12,7]. The bacterial culture media most commonly used to grow bacteria is Tryptic Soy Broth (TSB). This is because the content contained in TSB - such as casein, soybean peptone, dextrose and sodium chloride - can support the life of bacteria. However, the price of TSB is less economical, so solutions must be found that can reduce the use of this material. Based on the composition of the TSB, it is necessary to find alternative media that can be used for bacterial culture.

Some of the media that can be used as a bacterial culture media include molasses, kaolin and skimmed milk. Skimmed milk contains a high enough level of protein at 35% [13]. This protein content is broken down by bacteria into nitrogen elements, which will be used by bacteria as a source of nutrients for cell metabolism. Liquid whey tofu waste is the result of waste from tofu processing that cannot be consumed by humans. It is therefore necessary to make efforts to reduce its impact on the environment. Liquid whey tofu waste is often disposed of directly without processing in advance, which produces a foul odor and pollutes the environment [14].

Liquid whey tofu waste also contains phosphate compounds (P_2O); 228, or 85% [15]. Phosphate is a mineral that can support bacterial growth. This is because the phosphate will be broken down by bacteria into phosphorus (P) and used by the bacteria for cell formation. Therefore the available phosphorus will affect the growth rate of the bacteria [16]. Based on this background, the underlying research needs to be carried out on both materials that are to be used as bacterial culture media.

The purpose of this study was to determine the growth of *Flavobacterium* sp. and *Bacillus* sp. grown in a skimmed milk medium with the addition of liquid whey tofu waste. We also want to learn of the best concentration of liquid waste that is able to support the growth of *Flavobacterium* sp. and *Bacillus* sp.

2. Materials and methods

2.1. Place and time

This study was conducted from July to August 2017 in the Faculty of Fisheries and Marine Education Laboratory of Universitas Airlangga, Surabaya and at the Institute of Tropical Diseases of Universitas Airlangga.

2.2 Tools and materials

The materials used in this study included skimmed milk, *Flavobacterium* sp, *Bacillus* sp. and liquid whey tofu waste. The tools used were an autoclave, vortex, hand gloves, hot magnetic stirrer, measuring cup, refrigerator and an analytical scale.

2.3 Research design

This research was conducted using a completely randomized design (CRD) with four treatments and five replications. The treatments used in this study were: 10% TSB plus 10% Skim milk with 6% Glucose and 0% liquid whey tofu waste(P0), 10% TSB plus 10% Skim milk with 6% Glucose and 10% liquid whey tofu waste (P1), 10% TSB plus 10% Skim milk with 6% Glucose and 20% liquid whey tofu waste (P2), 10% TSB plus 10% Skim milk with 6% Glucose and 30% liquid whey tofu waste(P3). All of the treatments were given a 1.5% NaCl solution until the volume of the culture medium reached 10 ml.

2.4 Research preparation

2.4.1 Making TSB media

The preparation of TSB was carried out by dissolving 30 g in 1 liter of distilled water before heating it on a hot plate until it was homogeneous (9). Once the media was homogeneous, it was then sterilized using an autoclave at 121ºC for 15 minutes with a pressure of 1 atm.

2.4.2 Making skim milk media

We dissolved 100 grams of skim milk powder into 1 liter of distilled water before heating it on the hot plate until it was homogeneous. The homogeneous skim milk was sterilized using the water blanching method at 70ºC for 10 minutes.

2.4.3. Preparation of the liquid whey tofu waste

The liquid whey tofu waste was obtained from the tofu factory of PacarKeling, Surabaya. The liquid whey tofu waste used was the liquid waste generated by the tofu-making process. The liquid whey tofu waste was filtered using filter paper in order to separate the tofu waste from the water. This was then sterilized using an autoclave with a temperature of 121ºC for 15 minutes at a pressure of 1 atm.

2.4.4. *Media mixing for Bacillus* sp. *and Flavobacterium sp*.

Flavobacterium sp. and *Bacillus* sp. were put into a test tube with a volume of 20 ml with the media filling only half of the total volume (10 ml). The media mixing was done in the following ways:

P0 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml glucose + 7,4 ml NaCl solution 15 ppt

P1 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml glucose + 1 ml liquid whey tofu waste+ 6,4 NaCl solution 15 ppt

P2 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml glucose + 2 ml liquid whey tofu waste+ 5,4 ml NaCl solution 15 ppt

P3 : 1 ml Media TSB + 1 ml skim milk + 0,6 ml glucose + 3 ml liquid whey tofu waste + 4,4 ml NaCl solution 15 ppt

2.4.5 Inoculation and incubation of Flavobacterium sp. and Bacillus sp.

Flavobacterium sp. and *Bacillus* sp. were taken from the TSB media which had been calculated using a spectrophotometer with a wavelength [17] of 550 nm. The *Flavobacterium* sp. and *Bacillus* sp. were taken, as much as 1 ml, and put into each treatment. The results of the inoculation were incubated for 48 hours at 30-35ºC.

2.4.6 *Calculation of the bacterial growth amounts*

The calculation of the bacterial growth in this study was carried out by counting the bacterial colonies using the Total Plate Count (TPC) method. Based on the preliminary research, the calculation was done at dilutions of 10-7 and 10-8. The number of bacteria was calculated during the incubation period at 1, 6, 12, 18, 24, 30, 36, 42 and 48 hours respectively [24]. The formula for calculating the amount of bacteria using the TPC method according to Waluyo [18] is as follows:

$$\text{Number of Bacteria (CFU per ml)} = \text{number of colonies per dish} \times \frac{1}{\text{Dilution factor}}$$

2.4.7 Specific Growth Rate of bacteria

According to [19], the specific bacterial growth rates were calculated using the following formula:

$$\mu = \frac{(\ln Nt - \ln No)}{t_n - t_o}$$

μ = Specific growth rate
N = Number of bacteria at t_n
No = Number of bacteria at t_o
t_n = time at the n
t_o = time at the early

The data from the number of *Flavobacterium* sp. and *Bacillus* sp. was calculated using the TPC method and then analyzed using ANOVA (Analysis of variance) in order to determine the effect of the treatment given. From the analysis, it is known that the treatment showed a significantly different effect and then the researcher continued with Duncan's test in order to find out the difference between the treatments.

3 Results and discussion

3.1 Results

The growth data of *Bacillus* sp. from those cultured in the skim milk media with the addition of liquid whey tofu waste has been presented in Table 1.

Table 1. Average number of cells of *Bacillus* sp.

Treatment	The hour- (CFU/ml)									
	0	1	6	12	18	24	30	36	42	48
P0	10^5	17.92×10^{7a}	28.19×10^{7a}	62.89×10^{7a}	68.00×10^{7a}	31.65×10^{8a}	39.89×10^{8a}	42.64×10^{8a}	50.08×10^{8a}	52.40×10^{8a}
P1	10^5	17.05×10^{7a}	27.25×10^{7a}	46.35×10^{7a}	98.25×10^{7a}	59.24×10^{8a}	64.84×10^{8a}	78.46×10^{8b}	96.81×10^{8b}	12.08×10^{9b}
P2	10^5	7.85×10^{7a}	18.73×10^{7a}	32.00×10^{7a}	63.46×10^{7a}	26.52×10^{8a}	33.77×10^{8a}	43.01×10^{8a}	60.66×10^{8a}	10.21×10^{9b}
P3	10^5	6.68×10^{7a}	13.98×10^{7a}	65.21×10^{7a}	168.7×10^{7a}	50.27×10^{8a}	64.70×10^{8a}	71.58×10^{8b}	95.58×10^{8b}	10.54×10^{9b}

Description: P0: 0% liquid whey tofu waste, P1: 10% liquid whey tofu waste, P2: 20% liquid whey tofu waste, P3: 30% liquid whey tofu waste. Notations shown with different superscript letters in the same column show significant differences ($p < 0.05$).

The *Bacillus* sp. cultured in the medium with the addition of 10% liquid whey tofu waste (P1) showed a higher growth increase compared to the 20% and 30% additions. The treatment of P1 began to increase from the 18th hour and reached the exponential optimum point at the 48th hour, with the number of bacteri cells reaching 12.08 x 10^9 CFU/ml.

The treatment media with the addition of 20% liquid whey tofu waste (P2) and the treatment with the addition of 30% liquid whey tofu waste (P3) showed an increase in the number of bacteria that was lower than P1. The growth of *Bacillus* sp. in P2, starting from the first hour and up to 48 hours, reached 10.21 x10^9 CFU / ml in the bacterial count while in the P3, the number of *Bacillus* sp. reached the exponential optimum point at 48 hours with a bacterial cell count of 10.54 x 10^9CFU / ml.

In this study, the number of cells of *Flavobacterium* sp. was less than *Bacillus* sp. The data from the growth of *Flavobacterium* sp. cultured in the skim milk media with the addition of liquid whey tofu waste can be seen in Table 2.

Table 2 Average number of *Flavobacterium* sp. cells

Treatment	The hour- (CFU/ml)									
	0	1	6	12	18	24	30	36	42	48
P0	10^5	$0.52x10^{7a}$	$3.25x10^{7a}$	$6.67x10^{7a}$	$14.67x10^{7a}$	$37.11x10^{7a}$	$81.23x10^{7a}$	$93.91\ x10^{7a}$	$10.86\ x10^{8a}$	$12.01x10^{8a}$
P1	10^5	$0.28x10^{7a}$	$2.55x10^{7a}$	$5.72x10^{7a}$	$21.26x10^{7a}$	$88.00x10^{7b}$	$13.85\ x10^{8b}$	$15.20\ x10^{8b}$	$16.36\ x10^{8b}$	$18.25x10^{8b}$
P2	10^5	$0.52x10^{7a}$	$4.68x10^{7a}$	$11.81x10^{7a}$	$21.58x10^{7a}$	$64.36x10^{7ab}$	$11.46x10^{8ab}$	$13.71x10^{8b}$	$14.16x10^{8ab}$	$14.45x10^{8ab}$
P3	10^5	$0.44x10^{7a}$	$6.16x10^{7a}$	$15.74x10^{7a}$	$21.03x10^{7a}$	$49.00x10^{7ab}$	$95.14x10^{7ab}$	$11.55x10^{8ab}$	$12.36x10^{8ab}$	$13.13x10^{8a}$

Description: P0: 0% liquid whey tofu waste, P1: 10% liquid whey tofu waste, P2: 20% liquid whey tofu waste, P3: 30% liquid whey tofu waste. Notations shown with different superscript letters in the same column show significant differences ($p < 0.05$).

The growth of *Flavobacterium* sp. in the medium with the addition of 10% liquid whey tofu waste (P1) tended to be stable and increased well. This was shown in the first-hour of the observation; *Flavobacterium* sp. had a smaller growth increase than the other treatments. The growth of *Flavobacterium* sp. began with a significant increase in the 18th hour and reached the optimum point of the exponential phase at 48 hours, with the number of bacteria being 18.25 x 10^8 CFU / ml.

The treatment of P2 with the addition of liquid waste experienced a 20% increase in the number of bacterial cells which continued to increase from the first hour and reached the optimum point of the exponential phase at 48 hours with the number of bacterial cells being 14.45 x 108 CFU / ml. For treatment of P3 the growth of the number of bacterial cells tended to be slower compared to P0, P1, and P2. The optimum point of the exponential phase for *Flavobacterium* sp. was in tP3, which occurred at 48 hours with the number of bacterial cells being as much as 13.13 x 10^9 CFU / ml.

Bacillus sp. (Table 1) and *Flavobacterium* sp. (Table 2) showed that both bacteria in the treatment grown in liquid whey tofu waste culture media (P1, P2, and P3) showed a higher growth increase than the control (P0). In P0, P1, P2 and P3, *Bacillus* sp. and *Flavobacterium* sp. showed that the optimum point of the growth phase was at 48 hours. The highest number of cells of *Bacillus* sp. and *Flavobacterium* sp. was achieved in treatment P1, with the number of *Bacillus* sp cells being as much as 12.08 x 10^9 CFU / ml and for *Flavobacterium* sp, it was as much as 18.25 x 10^8 CFU/ml.

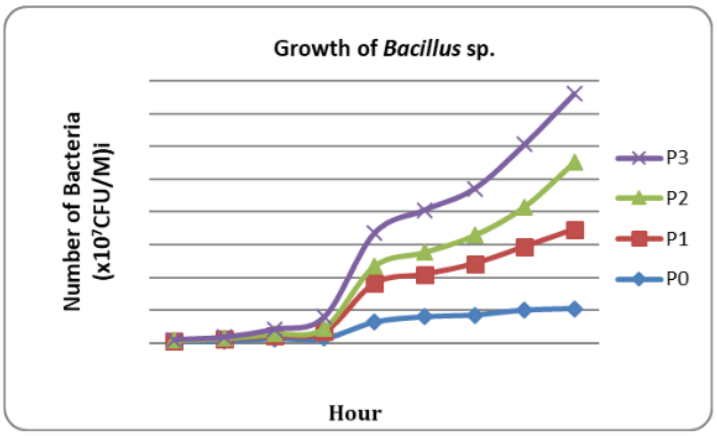

Figure 1. Growth of *Bacillus* sp.

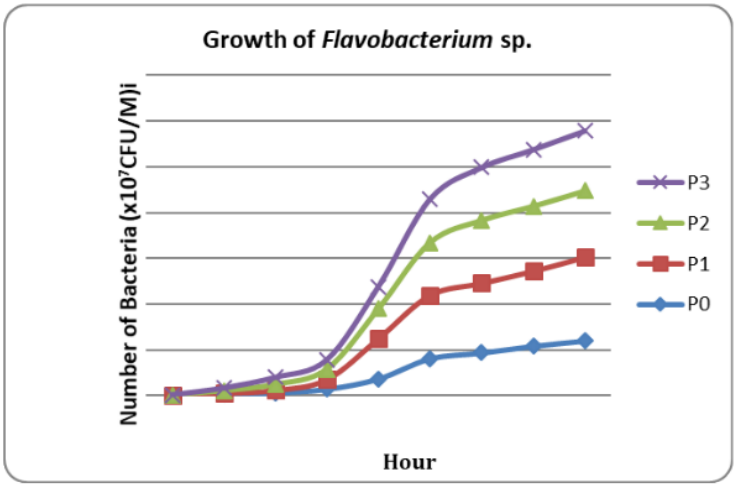

Figure 2. Growth of *Flavobacterium* sp.

Based on Figure 1, it appears that *Bacillus* sp. grown in the medium of adding liquid whey tofu waste had a phase of adaptation (lag) from 0 to the first hour. The logarithmic growth phase of both bacteria began after 6 to 36 hours. The growth of *Flavobacterium* sp. underwent a phase of adaptation (lag) at 0 to before 6 hours, and increased in the exponential phase from the 6th to 36th hours.
The growth rate of both bacteria grown in the medium of adding liquid whey tofu waste can be seen in Table 3.

Table 3. Specific growth of *Bacillus* sp. and *Flavobacterium* sp at exponential phase

Bacteria	Treatment			
	P0	P1	P2	P3
Bacillus sp.	0.09	0.11	0.10	0.13
Flavobacterium sp.	0.11	0.13	0.11	0.09

Both bacteria cultured on a culture media with the addition of 10% tofu wastewater had a better growth velocity compared to the growth rate of the bacterial cultured on the treatment medium with the addition of liquid waste at 0% and 20%.

Bacillus sp. in P1 had a specific growth rate of 0.11. This specific growth rate is higher than the P0 treatment, which was 0.09. P2 was 0.10. The *Flavobacterium* sp. in the P1 culture had a specific growth rate of 0.13. This specific growth rate was greater than P0 at 0.11, P2 at 0.11 and P3 at 0.09.

3.2 Discussion

Bacterial culture media is a material consisting of a mixture of nutrients used to grow microorganisms. The types of media based on their composition consisted of natural media, semi-synthetic media and synthesis media. Natural media is a medium composed of natural ingredients [8]. The culture media for *Bacillus* sp. and *Flavobacterium* sp. grown in this study used natural media by adding liquid whey tofu waste. Liquid whey tofu waste contains N 0.27%, P_2O_5 228.85%, K_2O 0.29% and 1.68% protein [15].

Bacillus sp. was cultured in skim milk media with the addition of 10% (P1), 20% (P2) and 30% (P3) liquid whey tofu waste at 12 hours, which showed a different growth rate than the control treatment (P0). The number of *Bacillus* sp. cells cultured at P1, P2 and P3 was higher than in P0 (control). The number of *Bacillus* sp. at P1, P2 and P3 was because, in this media, there was the addition of liquid whey tofu waste. Some bacteria such as *Enterobacter gergoviae* and *Bacillus* sp. are

known to grow in tofu waste water media [19]. In this study the addition of liquid whey tofu waste given to culture media was found to also increase the growth of *Bacillus* sp.

Bacillus sp. cultured with the addition of 10% tofu wastewater (P1) showed a higher number of cells (12.08 × 10^9 CFU/ml) that was more stable than those cultured in 20% liquid whey tofu waste (P2) and 30% (P3). This is presumably because the Nitrogen (N) and Phosphorus (P) content contained in the liquid waste can meet the needs of N and P in *Bacillus* sp. While the addition of 20% and 30% liquid whey tofu waste contains excess N and P, the *Bacillus* sp. is already unable to absorb the available N and P. *Bacillusthuringiensis* grown in a medium containing sufficient amino acids can increase cell growth, although if too much amino acid is present in the media, then it can cause low bacterial growth [20].

The element P (phosphorus) plays an important role in the formation of nucleic acids and phospholipids in bacteria (21). The element P in liquid whey tofu waste is P_2O_5 (phosphate), which is 228.85 ppm [15]. Phosphate is needed by bacteria as a component of ATP, nucleic acids and a number of coenzymes such as NAD, NADP and flavin. A lack of P (phosphorus) or excess P in a culture medium can slow down the bacterial growth process [22,21].

The addition of 10% (P1), 20% (P2) and 30% (P3) of liquid whey tofu waste in this study also affected the growth of *Flavobacterium* sp. This can be seen in the *Flavobacterium* sp; those who were cultured in all three treatments had higher cell numbers and growth rates than the control (P0). For the *Flavobacterium* sp. in P1, the number of cells was higher compared to P2 and P3, which was as much as 18.25 × 10^8 CFU / ml. *Flavobacterium* sp. in the additions 20% (P2) and 30% liquid whey tofu waste (P3) showed lower growth; the concentration of nutrients was so excessive so that the cells of *Flavobacterium* sp. were unable to utilize the nutrients in the media. Media containing a lot of nutrients causes its concentration to rise and it becomes hypertonic. Hypertonic solutions (high nutrient levels) can inhibit bacterial growth because they cause plasmolysis in the bacterial cell [19].

Bacillus sp. and *Flavobacterium* sp. which are grown together in the medium of skim milk with the addition of liquid whey tofu waste showed a different growth. The specific growth rate of *Bacillus* sp. with the addition of liquid whey tofu waste up until the 30% dose was higher than P0 (control), while for the *Flavobacterium* sp. at a 10% dose (Table 3), the *Bacillus* sp. growth was higher than *Flavobacterium* sp. The differences in the growth between *Bacillus* sp. and *Flavobacterium* sp. can be seen in Table 1 and Table 2. Differences in the number of bacteria between *Bacillus* sp. and *Flavobacterium* sp. cultured together were thought to be due to the nature of *Flavobacterium* sp. being non-fermentative [4], while *Bacillus* sp. is fermentative [23].

The properties of *Bacillus* sp. classified as fermentative bacteria, makes the bacteria able to utilize the nutrients in the liquid waste in both aerobic and anaerobic conditions. *Bacillus* sp. is anaerobic and a facultative anaerobic bacterium capable of growing in liquid whey tofu waste because it is capable of producing extracellular enzymes decomposing cellulose and hemicellulose, so therefore it is commonly found in liquid whey tofu waste [24].

Bacillus sp. is a fermentative bacterium, which is a group of bacteria that can produce energy in anaerobic conditions [23]. The use of nutrients in the media is carried out by *Bacillus* through oxidation-reduction reactions to produce the energy needed for its metabolism [18]. The results of this study indicate that although there are differences in the number of bacteria between *Bacillus* sp. and *Flavobacterium* sp., these two bacteria can grow together in one medium. This can be seen from the growth of both bacteria, which continued to increase up to 48 hours.

Bacterial culture in the medium with the addition of liquid whey tofu waste has an effect on the growth rate of the bacteria. This is because liquid whey tofu waste contains the phosphate needed by bacteria for growth and development.

4. Conclusion

Flavobacterium sp. and *Bacillus* sp. cultured in skim milk media with the addition of liquid whey tofu waste can grow together in the same medium. The optimum growth of *Flavobacterium* sp. and *Bacillus* sp. occurs in the medium with the addition of 10% liquid whey tofu waste and the growth of

Bacillus sp. occurs more than *Flavobacterium* sp. The optimum point of the exponential phase occurred at the 48th hour with the number of *Bacillus* sp. being 12.08×10^9 CFU / ml and *Flavobacterium* sp. being 18.25×10^8 CFU / ml.

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